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Attenuation of iNOS and COX2 by blueberry polyphenols is mediated through the suppression of NF-κB activation

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ABSTRACT

Treatment of BV2 microglial cells with blueberry extracts has been shown to be effective in reducing lipopolysaccharide (LPS)-induced proinflammatory mediators such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), inducible NO synthase (iNOS), and cyclooxygenase 2 (COX2). The current study explored the possibility that the down-regulation of iNOS and COX2 by blueberry extracts was mediated through NF-κB signaling pathway. A column-purified fraction of polyphenol-enriched blueberry extract (PC18) was used to treat LPS-activated BV2 cells. The results thus far showed that blueberry polyphenols significantly suppressed iNOS and COX2 promoter activities. In addition, blueberry polyphenols inhibited NF-κB nuclear translocation in LPS-activated BV2 cells. These findings suggested that the beneficial effects of blueberries may involve direct modulation of oxidative stress and/or inflammatory signaling cascades.

1. Introduction

Accumulating evidence indicates that inflammation in the central nervous system (CNS) increases during normal aging and that neuroinflammation is augmented by age-related neurodegenerative diseases (Eikelenboom & Veerhuis, 1996; O'Banion & Finch, 1996; Rozovsky et al., 1998). Microglial cells, which constitute up to 20% of the neuronal cell population in certain brain regions, have been shown to be activated during neuroinflammation (Dobrenis, 1998; Lawson et al., 1990). Chronic and sustained activation of microglial cells is attributed to enhanced signal transduction (induction of nuclear factor-kappa B, NF-κB) leading to the activation of inflammatory enzymes such as the inducible nitric oxide synthase (iNOS) and the inducible cyclooxygenase-2 (COX2) as well as cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Klein & Ackerman, 2003; Mrak & Griffin, 2005; Shukitt-Hale et al., 2008; Talley et al., 1995). Moreover, activated microglial cells produce high concentrations of reactive oxygen species resulting in an increased burden of oxidative stress on neuronal cells further contributing to the progression of neuroinflammation (Darley-Usmar et al., 1995; McGeer & McGeer, 2004). In fact, several neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease have been associated with oxidative stress-mediated neuroinflammation (McGeer & McGeer, 1998).

Plants produce a variety of phytochemicals which are not required *per se* for their primary cellular functions. These so-called secondary compounds, however, are involved in a wide array of ecological functions to enhance the survival of plants during environmental stress (Winkel-Shirley, 2002). Mounting evidence suggests that the health benefits observed for the consumption of a diet rich in fruit and vegetables are mainly due to the presence of these phytochemicals (Liu, 2003). Polyphenols, which are found in abundance in diets rich in fruit and vegetables, are bioactive compounds with antioxidant,

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anti-inflammatory, and anticarcinogenic properties (Surh et al., 2001). Blueberries are high in antioxidant activity as measured by the oxygen radical absorbance capacity assay (ORAC) (Cao et al., 1996; Prior et al., 1998). Blueberries and other polyphenolic-enriched fruit and vegetables have been shown to provide protection against oxidative stress, inflammation, carcinogenesis and chronic diseases (Graf et al., 2005; Lau et al., 2005b; McDougall et al., 2008; Schmidt et al., 2006).

Research has suggested that dietary supplementation with fruit and vegetables rich in polyphenolics is capable of both forestalling and reversing the deleterious effects of aging on neuronal communication and behavior (Lau et al., 2005a). Although the exact mechanisms for the observed beneficial effects are largely unclear, several lines of evidence suggests that the antioxidant and anti-inflammatory properties of the polyphenolic compounds found in these fruit and vegetables may play an important role (Rice-Evans et al., 2000; Rice-Evans & Miller, 1996).

Previously we have shown that crude extract of blueberry is effective in attenuating the production of inflammatory mediators in lipopolysaccharide (LPS) activated murine BV2 microglia (Lau et al., 2006). The current study aimed to determine the effect of a polyphenolic-enriched fraction of blueberry (PC18) on the production of proinflammatory mediators COX2 and iNOS and whether PC18 could affect nuclear factor-kappa B (NF-κB) translocation into the nucleus of BV2 microglia that were activated by the proinflammatory inducer lipopolysaccharide (LPS).

2. Materials and methods

2.1. Reagents

Dimethylsulphoxide (DMSO) and LPS (*Escherichia coli* serotype O111:B4) were purchased from Sigma (St. Louis, MO). Cell culture media, antibiotics, and serum were obtained from Invitrogen (Carlsbad, CA). Cell culture dishes and multi-well plates were supplied by Fisher Scientific (Pittsburgh, PA). Electrophoretic apparatus and reagents were purchased from Bio-Rad (Hercules, CA).

2.2. Cell culture

The BV2 murine microglial cell line (Blasi et al., 1990) was generously provided by Dr. Van Eldik of Northwestern University (Chicago, IL). BV2 cells were cultured at 37 °C in a humidified incubator under 5% CO_2 in growth medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), and referred as complete medium. For all colorimetric and luciferase-reporter experiments, cells were washed twice with warm phosphate-buffered saline (PBS) and incubated in warm serum-free DMEM in the absence of phenol red and antibiotics at the indicated durations.

2.3. Blueberry PC18 fraction preparation

A blueberry polyphenolic-enriched fraction (PC18) was prepared from a commercially prepared 100% wild blueberry (*Vaccinium angustifolium*) juice as described by (Wilson et al., 2006). Briefly, blueberry juice was applied to a pre-conditioned C18 column (Waters Canada Ltd., Mississauga, ON, Canada) and washed extensively with 0.1% trifluoroacetic acid (TFA) in water to remove abundant sugars and organic acids. Bound polyphenolics were eluted from the C18 column with 0.1% TFA in methanol. The acidified methanol was removed at 30 °C using a vacuum rotary evaporator (Buchi, Essen, Germany) and the remaining residue was lyophilized. Anthocyanins, which are the most abundant flavonoid polyphenolic in the PC18 fraction, were separated and identified by high performance liquid chromatography (HPLC).

2.4. HPLC elution

The PC18 fraction was analysed using an Agilent 1100 series HPLC (Agilent Technologies Ltd., Mississauga, ON), equipped with a binary pumping system, column compartment and diode array detection (DAD) at 280 and 520 nm. The column was a Zorbax-SB-C18 (Agilent Technologies Ltd, Mississuaga, ON) 2.1 × 100 mm 3.5 μm particle size maintained at 26 °C. Solvent A was TFA in water at pH 1.35, and Solvent B (SB) was TFA in acetonitrile at pH 1.35. The flow was 0.4 mL/min and the gradient elution program was as follows: 5% SB initial conditions; 5–10% SB, 0–25 min; 10–20% SB, 25–87.5 min; 20% SB, 87.5–90 min; 100% SB, 90–99 min; 100–5% SB, 99–102 min; 5% SB, and 102–105 min. The column was equilibrated for an additional 5 min prior to each run.

2.5. Cell viability assay

The CellTiter 96® AQ_{ueous} One Solution Assay (Promega Corp., Madison, WI) was used to determine whether the PC18 concentrations used in the experiment caused any cytotoxicity in BV2 cells. This assay is based on the mitochondrial mediated reduction of a tetrazolium compound (MTS) by living cells to form a colored formazan product which is measured colorimetrically (Cory et al., 1991; Malich et al., 1997). Briefly, BV2 cells (4×10^4 cells in 200 μl/well) were seeded for 24 h in 96-well plates. Cells were washed twice with PBS and then treated with or without PC18 at the specified concentrations in the presence of LPS (100 ng/ml) in 100 μl cell culture medium that did not contain phenol-red or antibiotics. After 16 h, 20 μl of MTS reagent were added to each well and the plates were returned to the incubator. After 4 h incubation, concentration of the MTS formazan product was measured at 490 nm in a 96-well μQuant microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). The assay was performed in quadruplicates. The absorbance values of PC18-treated cells was calculated relative to the values of PBS-treated control and reported as% cell viability.

2.6. iNOS activity

To assess the activity of iNOS, the concentration of extracellularly released nitrite (a stable metabolite of nitric oxide, NO) in cell-conditioned media (CCM) was measured by Griess reagent (Promega Corp., Madison, WI) according to the instructions provided by the manufacturer with slight modifications. Briefly, BV2 cells (2×10^5 cells in 500 μl/well) were seeded in

12-well tissue culture treated plates and allowed to grow for 24 h in complete growth medium at 37 °C and 5% CO_2. Cells were then washed twice with PBS and then pre-treated for 45 min with the blueberry PC18 fraction at specified concentrations in serum-free, phenol red-free medium. After pre-treatment, LPS (100 ng/ml) was added and the cells were incubated at 37 °C and 5% CO_2 for 16 h. After LPS exposure, CCM were collected. Triplicates of supernatants (100 μl/well) were mixed with 50 μl/well of Griess reagent in a 96-well plate and incubated at room temperature for 10 min in the dark. The absorbance was measured at 548 nm using a μQuant microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). Sodium nitrite standard curve with nitrite concentrations ranging from 0 to 100 μM. The concentration of nitrite released in the CCM was calculated with the linear equation derived from the standard curve.

2.7. *Immunoblot analysis*

Cell culture conditions and treatments for iNOS and COX2 immunoblot were identical to those in iNOS activity measurement using nitrite detection. After CCM were collected for nitrite measurements, cells were washed twice with ice-cold PBS before protein extraction. Protein was extracted using 100 μl/well of CelLytic-M mammalian cell lysis/extraction reagent (Sigma, St. Louis, MO) at 4 °C with rocking for 15 min followed by centrifugation at 12,000*g* for 15 min at 4 °C. Cell lysates were collected and protein concentration was determined by the *DC* Protein Assay Kit according to the instructions furnished by the manufacturer (Bio-Rad, Hercules, CA). Equal amounts of denatured protein samples (10 μg per lane) were separated by 12% SDS–PAGE and electrophoretically transferred to PVDF membrane (Bio-Rad, Hercules, CA). Except for the primary antibodies, reagents for immunoblotting assay were from WesternBreeze Immunodetection Kits (Invitrogen, Carlsbad, CA). Primary antibody for COX2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and iNOS was purchased from Upstate Cell Signaling (Waltham, MA). Procedures for immunodetection were as outlined in the instruction manual for WesternBreeze Immunodetection Kit. Immuno-reactivity was visualized and captured by an EC^3 BioImaging system (UVP, Upland, CA). The optical density of the antibody-specific bands was analyzed by the LabWorks image acquisition and analysis software (Upland, CA). PVDF membrane was stripped and reprobed with antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) to ensure equal loading of protein samples.

2.8. *Plasmid construction*

The murine COX2 and iNOS promoter plasmids were purchased from Oxford Biomedical Research Inc. (Oxford, MI). The promoterless reporter vector (pGluc-Basic) containing the humanized coding sequence of the secreted Gaussia luciferase as well as enzymes used for restriction digestion and cloning were purchased from New England Biolabs, Inc. (NEB, Ipswich, MA). PCR primers flanking the multiple cloning site and upstream of the Gaussia luciferase (Gluc) gene were also obtained from NEB. Taq PCR Master Mix Kit was purchased from Qiagen (Valencia, CA). Competent cells of strain MJ109 were purchased from Promega Corp. (Madison, WI). The reporter plasmids were constructed using standard cloning techniques. Briefly, the COX2 promoter region (containing a 1004 bp fragment, −965 to +39 bp relative to the transcription initiation site) was excised by *Hind*III–*Eco*RV digestion, gel purified and then directionally cloned into the unique site of the *Hind*III–*Eco*RV linearized pGluc-Basic vector upstream of the Gluc gene resulting in the COX2-pGluc reporter construct. iNOS promoter region (containing a 1749 bp fragment, −1588 to +161 bp relative to the transcription initiation site) was excised by *Ale*I blunt-end restriction enzyme, gel purified and cloned into the *Eco*RV blunt-end linearized pGluc-Basic vector upstream of the Gluc gene. The orientation of iNOS promoter was verified by asymmetrical restriction digestion with *Sac*I enzyme resulting in the iNOS–pGluc reporter plasmid. The reporter plasmids were maintained and propagated in JM109 cells and purified by Qiagen Plasmid Maxi Kit according to the instructions supplied by the manufacturer (Qiagen, CA). The concentration of the plasmids was measured as described in the Quant-iT DNA Assay Kit by Invitrogen (Carlsbad, CA).

2.9. *Transient transfection and luciferase assay*

BV2 cells were seeded in 100 mm dishes in complete medium and incubated until the cells were confluent. The medium was replaced with serum-free Opti-MEM I medium (OMEM, Invitrogen, Carlsbad, CA). Then 60 μl of Lipofectamine 2000 reagent (Invitrogen) were diluted in 1.5 ml OMEM, mixed with 24 μg of reporter construct (COX2-Gluc or iNOS-Gluc) in 1.5 ml OMEM and incubated for 20 min at room temperature to allow the plasmid-Lipofectamine 2000 complexes to form. Confluent BV2 cells were transfected with the COX2-Gluc or iNOS–Gluc–Lipofectamine 2000 complexes. The medium was replaced after 6 h and the cells were incubated for 24 h prior to LPS activation. Transfected cells were trypsinized and equal number of cells were seeded in 24-well plates for 16 h. Cells were treated with LPS (100 ng/ml) and specified concentrations of PC18 for 4 h. CCM were collected for luciferase assay since the Gluc protein upon expression was secreted into the media. Gaussia Luciferase Assay Kit (NEB, Ipswich, MA) was used to determine the amount of COX2- or iNOS-induced reporter luciferase activity according to the manufacturer's instructions. Briefly, 20 μl of CCM were mixed with 40 μl of single strength Gaussia Luciferase Assay reagent. The luminescence was immediately measured in a LUMI-SCINT luminometer (Bioscan, Washington, DC) for 15 s/well following a 10 s delay in the dark. Luciferase activity was recorded as relative light units (RLU) and expressed as % relative to treatment-free control.

2.10. *Electrophoretic mobility-shift assay (EMSA)*

BV2 cells were seeded in 6-well plates and incubated until the cells were confluent. The media were replaced with serum-free DMEM and cells were treated with LPS (100 ng/ml) and specified concentrations of PC18 for 4 h. Nuclear proteins were isolated with CelLytic NuClear Extraction Kit (Sigma, St. Louis, MO) according to the technical bulletin supplied by the manufacturer. EMSA was performed according to manufacturer's instructions (Panomics, Redwood City, CA). Briefly, 10 μg of nuclear proteins were mixed with biotinylated

double-stranded oligonucleotide probe containing the NF-κB binding site. The protein–DNA complexes were separated on a 6% non-denaturing acrylamide gel and transferred to nylon membrane. The specificity of NF-κB nuclear protein–DNA binding was confirmed by competition with unlabeled oligonucleotide probes resulting in the absence of the shifted band. The labeled DNA probe was detected by streptavidin-HRP conjugate in the presence of chromogen substrate and visualized with the EC^3 BioImaging system (UVP, Upland, CA).

2.11. *Statistical analysis*

The results were expressed as mean ± standard error of mean (SEM) from at least three independent experiments. For multiple variable comparisons, data were analyzed by analysis of variance (ANOVA) followed by Tukey's *post hoc* pair-wise comparisons using SYSTAT program (SYSTAT Software Inc., Point Richmond, CA). The level of statistical significance was set a priori at $P < 0.05$.

3. Results

3.1. *Effect of blueberry PC18 fraction on cell viability and LPS-induced iNOS activity*

Total phenolics and anthocyanins present in the PC18 fraction were analyzed by HPLC. As illustrated in Fig. 1A, the major polyphenol in PC18 is chlorogenic acid. Figure 2A revealed the anthocyanins present in PC18 fraction. The tentative identities of the peaks were summarized in Table 1.

MTS assay was used to examine whether the concentrations of PC18 used in this study caused cytotoxicity in BV2 cell. As demonstrated in Fig. 2, concentrations of PC18 up to 100 μg/ml did not affect BV2 cell viability in the presence of LPS.

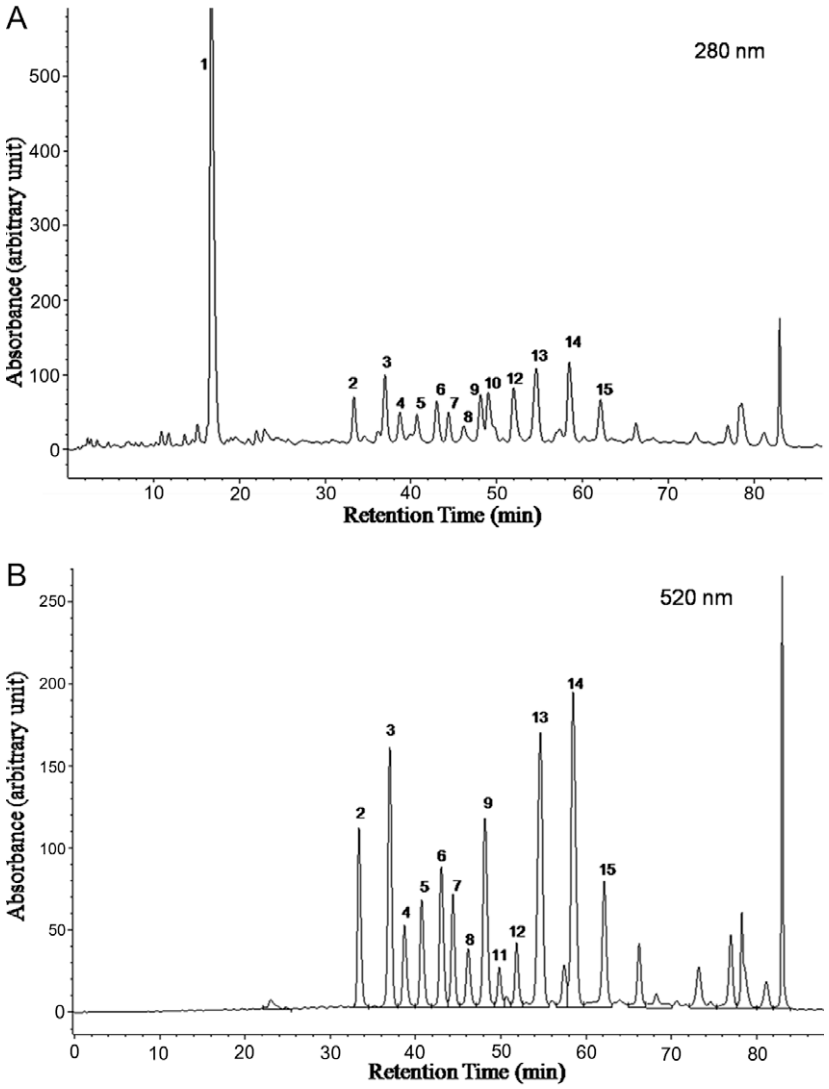

Fig. 1 – Chromatograms of total phenolics (A) and anthocyanins (B) found in blueberry PC18 fraction. Blueberry juice was fractionated by C18 column and washed with 0.1% trifluoroacetic acid (TFA) in water to remove abundant sugars and organic acids. Bound polyphenolics were eluted from the C18 column with 0.1% TFA in methanol. The acidified methanol was removed at 30 °C using a vacuum rotary evaporator and the remaining residue was lyophilized. Total phenolics and anthocyanins in the resulting PC18 fraction were identified by standard HPLC procedures and detected at 280 nm and 520 nm.

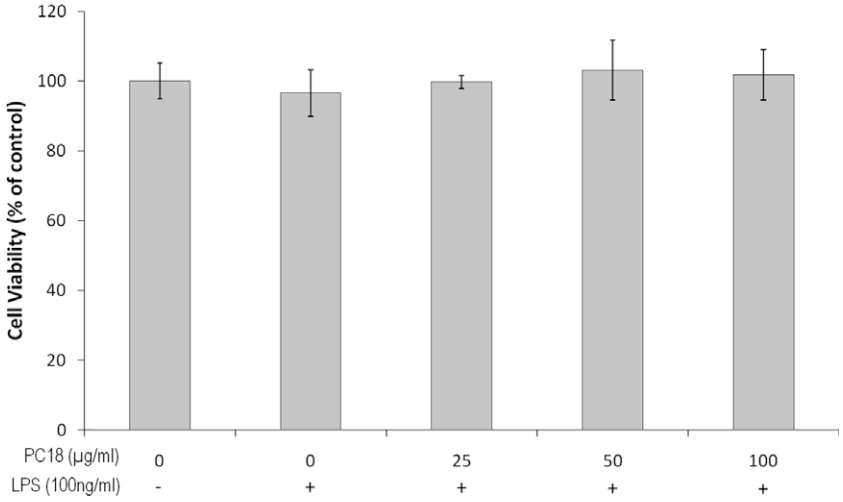

Fig. 2 – Effect of blueberry PC18 fraction on the viability of BV2 cells. BV2 cells were treated with or without LPS (100 ng/ml) in the presence or absence of PC18 at various concentrations for 16 h. Cell viability was measured colorimetrically by MTS assay. Data represented mean ± SEM of four independent experiments and were expressed as % of control data. No statistically significant difference in cell viability among the treatments was observed ($P > 0.3$).

Table 1 – Identities of phenolics and anthocyanins found in blueberry PC18 fraction.

Peak[a]	t_R (min)[b]	Tentative identity
1	16.73	Chlorogenic acid
2	33.374	Delphinidin-3-galactoside
3	36.988	Delphinidin-3-glucoside
4	38.712	Cyanidin-3-galactoside
5	40.728	Delphinidin-3-arabinoside
6	43.032	Cyanidin-3-glucoside
7	44.391	Petunidin-3-galactoside
8	46.177	Cyanidin-3-arabinoside
9	48.133	Petunidin-3-glucoside
10	49.029	Unknown
11	49.827	Peonidin-3-galactoside
12	51.857	Petunidin-3-arabinoside
13	54.638	Peonidin-3-glucoside and malvidin-3-galactoside
14	58.402	Malvidin-3-glucoside
15	62.137	Malvidin-3-arabinoside

a,b See Fig. 1 for peak number and t_R.

iNOS activity was assessed by the level of nitrite in the CCM (Griess, 1879; Phizackerley & Al-Dabbagh, 1983). The addition of LPS significantly stimulated BV2 cells causing a surge of nitrite to be released into the medium. Without LPS-activation, there was very little detectable nitrite in the CCM of the vehicle-control group (Fig. 3). However, LPS elicited a drastic increase in nitrite secretion by BV2 cells as indicated by the LPS-alone treatment group. Treatment of LPS-activated BV2 cells with PC18 significantly reduced the level of nitrite in the CCM in a dose-responsive manner (Fig. 3). It has been reported that LPS treatment of cultured microglial cells caused secretion of cytokines, activation of respiratory burst and induction of nitric oxide synthase (Chun et al., 2005; Zielasek & Hartung, 1996).

3.2. *Inhibitory effect of PC18 on COX2 and iNOS protein expression*

The levels of COX2 and iNOS protein were measured quantitatively by immunoblotting and normalized to the protein expression of internal control β-actin. COX2 and iNOS protein expression was hardly detectable in resting BV2 microglia. COX2 and iNOS protein levels were significantly stimulated after LPS-treatment (Fig. 4). This increase in COX2 and iNOS protein expression in BV2 cells after LPS activation was significantly reduced by PC18 treatment at concentrations of 50 and 100 μg/ml (Fig. 4). PC18 at a concentration of 100 μg/ml reduced COX2 protein expression to 50% and iNOS protein expression to 20% as compared to LPS treatment alone (Fig. 4).

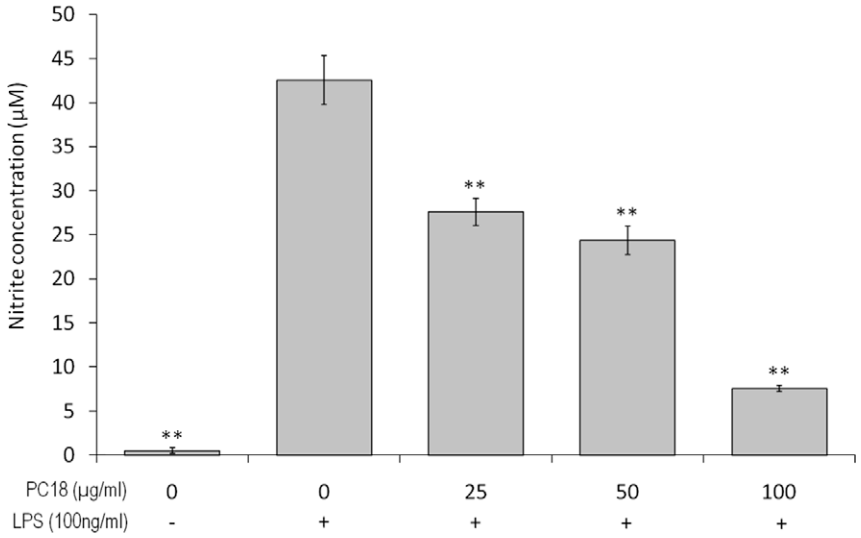

Fig. 3 – Inhibition of iNOS activity by blueberry PC18 fraction. BV2 cells were pretreated with PC18 at various concentrations as indicated, followed by activation with LPS (100 ng/ml) for 16 h. Cell-conditioned supernatant was collected and the concentration of nitrite in the supernatants was determined by Griess reagent. Values represent mean ± SEM of four independent experiments. $^{**}P < 0.001$ different from LPS-alone treatment group.

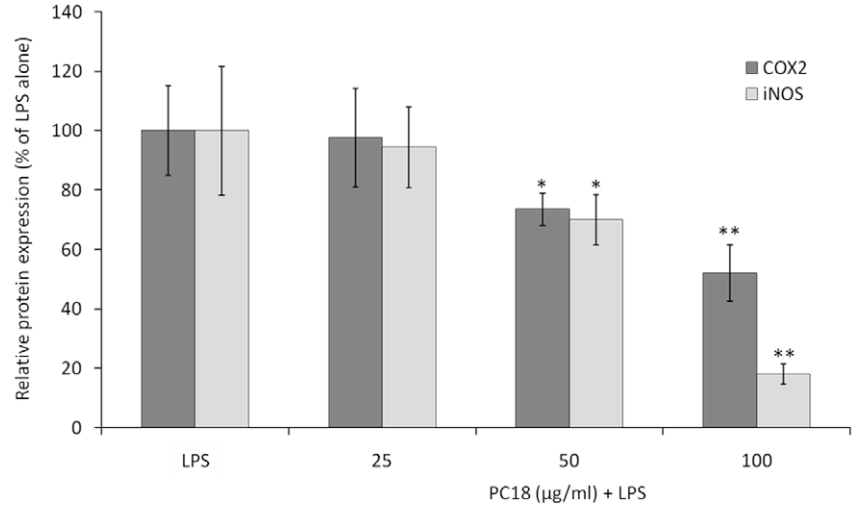

Fig. 4 – Suppression of LPS-induced COX2 and iNOS protein expression by blueberry PC18 fraction. BV2 cells were pretreated with PC18 at the concentrations indicated, followed by stimulation with LPS (100 ng/ml) for 16 h. Proteins from cell lysates were used for immunoblot analysis with the specified antibodies. The level of protein expression was expressed as % of that in the LPS-alone treatment group. Values represent mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.001$ different from LPS-alone treatment group.

3.3. *Suppression of LPS-activated COX2 and iNOS promoter activity by PC18 fraction*

To determine whether the alteration in COX2 and iNOS protein levels was due to changes in protein synthesis or protein degradation, modulation of COX2 and iNOS promoter activity by PC18 was investigated with COX2 and iNOS luciferase reporter assay. BV2 cells transiently transfected with COX2 or iNOS-luciferase promoter construct and activated with LPS had a three and twofold increase in COX2 and iNOS promoter activity, respectively (Figs. 5A and B). Treatment with PC18 significantly reduced the LPS-stimulated up-regulation of COX2 and iNOS promoter activities in a dose-responsive manner (Fig. 5A and B). This finding indicated that the inhibitory effect of PC18 on COX2 and iNOS protein expression was at the transcriptional level. The result also suggested that PC18 might act upstream of COX2 and iNOS transcriptional regulation by inhibiting the LPS-mediated signal transduction via NF-κB activation.

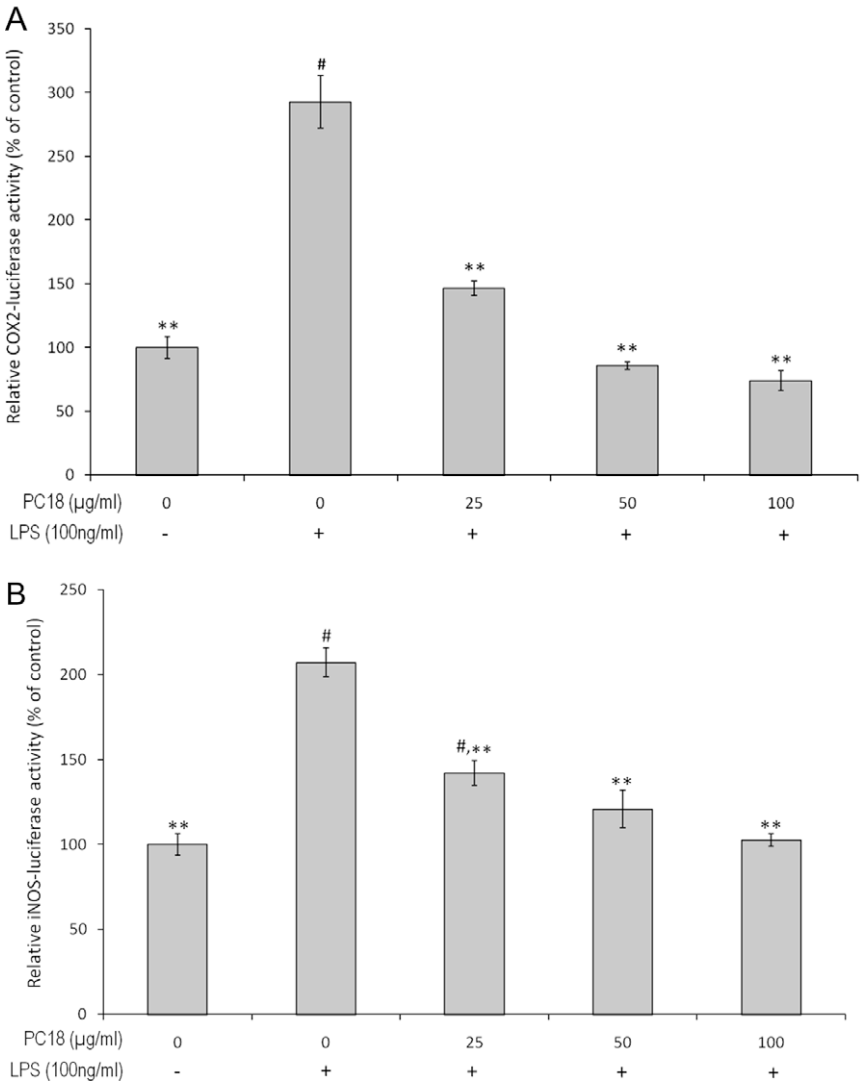

Fig. 5 – Effect of blueberry extract PC18 on LPS-induced COX2 and iNOS promoter activities. BV2 cells were transiently transfected with murine COX2 or iNOS promoter-luciferase reporter construct. Transfected cells were treated with LPS (100 ng/ml) and PC18 at specified concentrations for 4 h. Cell-conditioned supernatant was collected for the quantification of promoter activities of COX2 (A) and iNOS (B) by measuring relative light units. $^{*}P < 0.05$; $^{**}P < 0.001$ different from LPS-alone treatment group. $^{\#}P < 0.05$ different from control (no treatment) group.

3.4. *Inactivation of LPS-induced NF-κB nuclear translocation by blueberry PC18 fraction*

To evaluate the mechanism of PC18-mediated attenuation of COX2 and iNOS, EMSA was conducted to determine whether PC18 was able to suppress NF-κB nuclear translocation. After four hours of LPS stimulation, nuclear NF-κB protein–DNA binding activity was markedly increased as compared to the control (shifted bands, Fig. 6). Incubation with excess unlabelled NF-κB consensus oligo DNA abolished this shifted band indicating the specificity of nuclear NF-κB protein–DNA binding reaction (data not shown). Co-treatment with PC18 decreased the intensity of this shifted band suggesting that PC18 inhibited the translocation of NF-κB to the nucleus (Fig. 6).

4. Discussion

Previous research showed that blueberry supplementation effectively reduced proinflammatory mediators in both *in vitro* and *in vivo* experiments (Goyarzu et al., 2004; Lau et al., 2007; Shukitt-Hale et al., 2008). The results from the current study provided significant insight into the underlying mechanisms by which blueberry polyphenols modulate the expression of inflammatory cytokines. This study revealed that a blueberry polyphenolic-enriched fraction, PC18, significantly inhibited the LPS-induced production of proinflammatory mediators NO, iNOS and COX2 in BV2 microglial cell.

It has been shown that inflammation in the CNS produces oxidative stress through respiratory burst of activated microglia (Floyd & Hensley, 2002; Khanna et al., 2001; Rosen et al.,

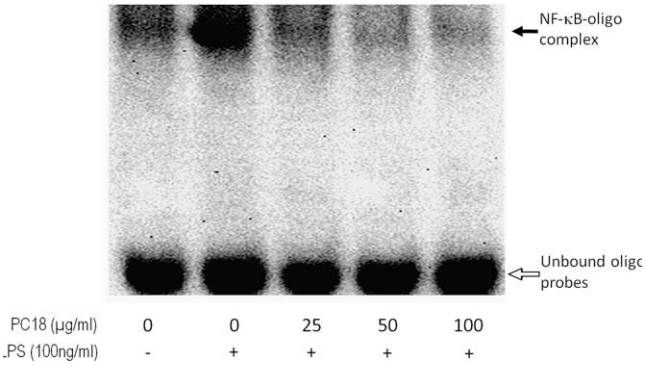

Fig. 6 – Inhibition of LPS-activated NF-κB nuclear protein–DNA activity by blueberry extract PC18. BV2 cells were treated with LPS and various concentrations of PC18 for 4 h. Nuclear proteins were extracted and used to assess the NF-κB nuclear protein–DNA binding activity by EMSA. Block arrow indicates shifted oligo-probe while open arrow denotes unbound oligo-probe.

1995). This process creates a vicious cycle through the conversion of neuroinflammation-induced oxidative stress to neurotoxic response which in turn further facilitates inflammation (Colton et al., 1994; Rosen et al., 1995). The inducible enzymes COX2 and iNOS as well as the second messenger NO have been shown to be the major factors in mediating inflammatory processes (Surh et al., 2001). Microglial activation is a well characterized hallmark of inflammation in the CNS (Liu et al., 2002). Chronic activation of microglia may lead to loss of neuronal cells thereby contributing to the progressive damage in neurodegenerative disorders including stroke, multiple sclerosis, Alzheimer's disease and Parkinson's disease (McGeer & McGeer, 2004; Orr et al., 2002). This pathological microglial activation is achieved through continued stimulation of inflammatory genes such as COX2 and iNOS as well as sustained production of various proinflammatory mediators including NO, ROS, and TNF-α (Akiyama et al., 2000; Luterman et al., 2000; Tarkowski et al., 2003).

The current findings indicated that polyphenols from blueberry extract significantly suppressed inflammation in LPS-activated BV2 microglia which appeared to indicate that polyphenol-enriched diet might be beneficial in alleviate inflammation associated with neurodegenerative diseases, insofar as these polyphenolic compounds are bioavailable in the brain. In fact, it has been demonstrated that polyphenolic compounds were able to cross the blood brain barrier and localized to various brain regions crucial for learning and memory (Andres-Lacueva et al., 2005). It is conceivable to postulate that the absorption and distribution of polyphenols in the brain have an important impact on their bioavailability and bioactivity. Indeed, a positive correlation was observed between Morris water maze performance and the total number of anthocyanin compounds found in the cortex of aged rats supplemented with blueberry extract. These findings strongly suggest that polyphenolic compounds are bioavailable and they deliver their beneficial effects such as antioxidant and signaling properties directly to the CNS thereby forestalling and even reversing the deleterious effects of aging on neuronal communication, memory, and behavior (Andres-Lacueva et al., 2005; Joseph et al., 2005).

Although the major focus of dietary phytochemicals has been on the antioxidant properties, it is apparent that these phytochemicals and their metabolite *in vivo* may not serve as conventional hydrogen-donating antioxidants. Rather, these dietary phytochemicals may exert their beneficial actions through modulation of signaling cascades (Williams et al., 2004). Polyphenols including flavonoids and their metabolites have been demonstrated to regulate phosphoinositide 3-kinase, Akt/protein kinase, tyrosine kinases, protein kinase C, and mitogen activated protein kinase signaling pathways (Williams et al., 2004). Corroborating with these findings, the current study demonstrated that blueberry polyphenols significantly suppressed COX2 an iNOS promoter activities. This suppression was probably upstream of COX2 and iNOS transcriptional regulation as indicated by the inhibitory action of blueberry polyphenols on NF-κB nuclear translocation. Therefore, blueberry polyphenols may act as modulators of NF-κB signal transduction. The nuclear factor NF-κB is an eukaryotic ubiquitous proinflammatory transcription factor which is sequestered in the cytoplasm under normal conditions by noncovalently bound to inhibitor IκB (Karin and Greten, 2005). Upon activation, NF-κB translocates into the nucleus to initiate the expression of over 200 immune, growth, and inflammation genes (Aggarwal, 2004). Here we showed that the inhibitory effects of blueberry polyphenols on COX2 and iNOS were due to the attenuation of NF-κB nuclear translocation. Thus the observed cell-signaling properties of blueberry polyphenols may be independent of their traditional antioxidant reducing activities.

Given that increased activation of NF-κB associated with the inflammatory processes is involved in the initiation and progression of diverse diseases, inhibition of NF-κB by blueberry polyphenols may present a powerful means for the development of anti-inflammatory nutraceuticals. In this regard, studies further explore the biomedical significance of blueberry polyphenols in the prevention and treatment of inflammation-related diseases are warranted.

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REFERENCES

Aggarwal, B. B. (2004). Nuclear factor-kappab: The enemy within. *Cancer Cell, 6*(3), 203–208.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., Cooper, N. R., Eikelenboom, P., Emmerling, M., Fiebich, B. L., Finch, C. E., Frautschy, S., Griffin, W. S., Hampel, H., Hull, M., Landreth, G., Lue, L., Mrak, R., Mackenzie, I. R., Mcgeer, P. L., O'banion, M. K., Pachter, J., Pasinetti, G., Plata-Salaman, C., Rogers, J., Rydel, R., Shen, Y., Streit, W., Strohmeyer, R., Tooyoma, I., Van Muiswinkel, F. L., Veerhuis, R., Walker, D.,

Webster, T., Wegrzyniak, B., Wenk, G., & Wyss-Coray, T. (2000). Inflammation and alzheimer's disease. *Neurobiology of Aging, 21*(3), 383–421.

Andres-Lacueva, C., Shukitt-Hale, B., Galli, R. L., Jauregui, O., Lamuela-Raventos, R. M., & Joseph, J. A. (2005). Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. *Nutritional Neuroscience, 8*(2), 111–120.

Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., & Bistoni, F. (1990). Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *Journal of Neuroimmunology, 27*(2–3), 229–237.

Cao, G., Sofic, E., & Prior, R. L. (1996). Antioxidant capacity of tea and common vegetables. *Journal of Agricultural and Food Chemistry, 44*, 3426–3431.

Chun, W., Lee, H. J., Kong, P. J., Lee, G. H., Cheong, I. Y., Park, H., & Kim, S. S. (2005). Synthetic wogonin derivatives suppress lipopolysaccharide-induced nitric oxide production and hydrogen peroxide-induced cytotoxicity. *Archives of Pharmacal Research, 28*(2), 216–219.

Colton, C. A., Snell, J., Chernyshev, O., & Gilbert, D. L. (1994). Induction of superoxide anion and nitric oxide production in cultured microglia. *Annals of the New York Academy of Sciences, 738*, 54–63.

Cory, A. H., Owen, T. C., Barltrop, J. A., & Cory, J. G. (1991). Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Communications, 3*(7), 207–212.

Darley-Usmar, V., Wiseman, H., & Halliwell, B. (1995). Nitric oxide and oxygen radicals: A question of balance. *FEBS Letters, 369*(2–3), 131–135.

Dobrenis, K. (1998). Microglia in cell culture and in transplantation therapy for central nervous system disease. *Methods, 16*(3), 320–344.

Eikelenboom, P., & Veerhuis, R. (1996). The role of complement and activated microglia in the pathogenesis of alzheimer's disease. *Neurobiology of Aging, 17*(5), 673–680.

Floyd, R. A., & Hensley, K. (2002). Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases. *Neurobiology of Aging, 23*(5), 795–807.

Goyarzu, P., Malin, D. H., Lau, F. C., Taglialatela, G., Moon, W. D., Jennings, R., Moy, E., Moy, D., Lippold, S., Shukitt-Hale, B., & Joseph, J. A. (2004). Blueberry supplemented diet: Effects on object recognition memory and nuclear factor-kappa b levels in aged rats. *Nutritional Neuroscience, 7*(2), 75–83.

Graf, B. A., Milbury, P. E., & Blumberg, J. B. (2005). Flavonols, flavones, flavanones, and human health: Epidemiological evidence. *Journal of Medicinal Food, 8*(3), 281–290.

Griess, P. (1879). Bemerkungen zu der abhandlung der hh weselsky und benedikt. "ueber einige azoverbindungen". *Chemische Berichte, 12*(42), 6–28.

Joseph, J. A., Shukitt-Hale, B., & Casadesus, G. (2005). Reversing the deleterious effects of aging on neuronal communication and behavior: Beneficial properties of fruit polyphenolic compounds. *The American Journal of Clinical Nutrition, 81*(1 Suppl), 313S–316S.

Karin, M., & Greten, F. R. (2005). Nf-kappab: Linking inflammation and immunity to cancer development and progression. *Nature Reviews. Immunology, 5*(10), 749–759.

Khanna, R., Roy, L., Zhu, X., & Schlichter, L. C. (2001). K+ channels and the microglial respiratory burst. *American Journal of Physiology. Cell Physiology, 280*(4), C796–806.

Klein, J. A., & Ackerman, S. L. (2003). Oxidative stress, cell cycle, and neurodegeneration. *The Journal of Clinical Investigation, 111*(6), 785–793.

Lau, F. C., Bielinski, D. F., & Joseph, J. A. (2006). Inhibitory effect of blueberry extract on the production of inflammatory mediators in lps-activated bv2 microglia. *Age, 28*(1), 46.

Lau, F. C., Bielinski, D. F., & Joseph, J. A. (2007). Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated bv2 microglia. *Journal of Neuroscience Research, 85*(5), 1010–1017.

Lau, F. C., Shukitt-Hale, B., & Joseph, J. A. (2005). Age-related neuronal and behavioral deficits are improved by polyphenol rich blueberry supplementation. In Y. Luo & L. Packer (Eds.), *Oxidative stress and age-related neurodegeneration* (pp. 373–393). CRC Press: Boca Raton.

Lau, F. C., Shukitt-Hale, B., & Joseph, J. A. (2005b). The beneficial effects of fruit polyphenols on brain aging. *Neurobiology of Aging, 26*(Suppl. 1), 128–132.

Lawson, L. J., Perry, V. H., Dri, P., & Gordon, S. (1990). Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience, 39*(1), 151–170.

Liu, B., Gao, H. M., Wang, J. Y., Jeohn, G. H., Cooper, C. L., & Hong, J. S. (2002). Role of nitric oxide in inflammation-mediated neurodegeneration. *Annals of the New York Academy of Sciences, 962*, 318–331.

Liu, R. H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition, 78*(3 Suppl.), 517S–520S.

Luterman, J. D., Haroutunian, V., Yemul, S., Ho, L., Purohit, D., Aisen, P. S., Mohs, R., & Pasinetti, G. M. (2000). Cytokine gene expression as a function of the clinical progression of alzheimer disease dementia. *Archives of Neurology, 57*(8), 1153–1160.

Malich, G., Markovic, B., & Winder, C. (1997). The sensitivity and specificity of the mts tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines. *Toxicology, 124*(3), 179–192.

Mcdougall, G. J., Ross, H. A., Ikeji, M., & Stewart, D. (2008). Berry extracts exert different antiproliferative effects against cervical and colon cancer cells grown in vitro. *Journal of Agricultural and Food Chemistry, 56*(9), 3016–3023.

Mcgeer, E. G., & Mcgeer, P. L. (1998). The importance of inflammatory mechanisms in alzheimer disease. *Experimental Gerontology, 33*(5), 371–378.

Mcgeer, P. L., & Mcgeer, E. G. (2004). Inflammation and the degenerative diseases of aging. *Annals of the New York Academy of Sciences, 1035*, 104–116.

Mrak, R. E., & Griffin, W. S. (2005). Glia and their cytokines in progression of neurodegeneration. *Neurobiology of Aging, 26*(3), 349–354.

O'banion, M. K., & Finch, C. E. (1996). Inflammatory mechanisms and anti-inflammatory therapy in alzheimer's disease. *Neurobiology of Aging, 17*(5), 669–671.

Orr, C. F., Rowe, D. B., & Halliday, G. M. (2002). An inflammatory review of parkinson's disease. *Prog. Neurobiol., 68*(5), 325–340.

Phizackerley, P. J., & Al-Dabbagh, S. A. (1983). The estimation of nitrate and nitrite in saliva and urine. *Analytical Biochemistry, 131*(1), 242–245.

Prior, R. L., Cao, G., Martin, A., Sofic, E., Mcewen, J., O'brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., & Mainland, M. (1998). Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity and variety of vaccinium species. *Journal of Agricultural and Food Chemistry, 46*, 2586–2593.

Rice-Evans, C., Spencer, J. P., Schroeter, H., & Rechner, A. R. (2000). Bioavailability of flavonoids and potential bioactive forms in vivo. *Drug Metabolism and Drug Interactions, 17*(1–4), 291–310.

Rice-Evans, C. A., & Miller, N. J. (1996). Antioxidant activities of flavonoids as bioactive components of food. *Biochemical Society Transactions, 24*, 790–794.

Rosen, P., Ballhausen, T., Bloch, W., & Addicks, K. (1995). Endothelial relaxation is disturbed by oxidative stress in the diabetic rat heart: Influence of tocopherol as antioxidant. *Diabetologia, 38*(10), 1157–1168.

Rozovsky, I., Finch, C. E., & Morgan, T. E. (1998). Age-related activation of microglia and astrocytes: In vitro studies show persistent phenotypes of aging, increased proliferation, and

resistance to down-regulation. *Neurobiology of Aging, 19*(1), 97–103.

Schmidt, B. M., Erdman, J. W., Jr., & Lila, M. A. (2006). Differential effects of blueberry proanthocyanidins on androgen sensitive and insensitive human prostate cancer cell lines. *Cancer Letters, 231*(2), 240–246.

Shukitt-Hale, B., Lau, F. C., Carey, A. N., Galli, R. L., Spangler, E. L., Ingram, D. K., & Joseph, J. A. (2008). Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. *Nutritional Neuroscience, 11*(4), 172–182.

Surh, Y. J., Chun, K. S., Cha, H. H., Han, S. S., Keum, Y. S., Park, K. K., & Lee, S. S. (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: Down-regulation of cox-2 and inos through suppression of nf-kappa b activation. *Mutation Research*, 243–268.

Talley, A. K., Dewhurst, S., Perry, S. W., Dollard, S. C., Gummuluru, S., Fine, S. M., New, D., Epstein, L. G., Gendelman, H. E., & Gelbard, H. A. (1995). Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: Protection by the antioxidant n-acetylcysteine and the genes bcl-2 and crma. *Molecular and Cellular Biology, 15*(5), 2359–2366.

Tarkowski, E., Liljeroth, A. M., Minthon, L., Tarkowski, A., Wallin, A., & Blennow, K. (2003). Cerebral pattern of pro- and anti-inflammatory cytokines in dementias. *Brain Research Bulletin, 61*(3), 255–260.

Williams, R. J., Spencer, J. P., & Rice-Evans, C. (2004). Flavonoids: Antioxidants or signalling molecules? *Free Radical Biology and Medicine, 36*(7), 838–849.

Wilson, M. A., Shukitt-Hale, B., Kalt, W., Ingram, D. K., Joseph, J. A., & Wolkow, C. A. (2006). Blueberry polyphenols increase lifespan and thermotolerance in caenorhabditis elegans. *Aging Cell, 5*(1), 59–68.

Winkel-Shirley, B. (2002). Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology, 5*(3), 218–223.

Zielasek, J., & Hartung, H. P. (1996). Molecular mechanisms of microglial activation. *Advances in Neuroimmunology, 6*(2), 191–222.